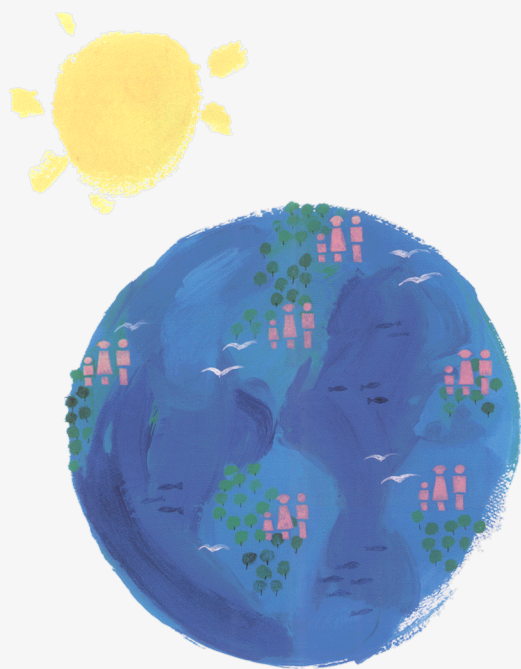


# NICEATM

*National Toxicology Program  
Interagency Center for the Evaluation of  
Alternative Toxicological Methods*

# ICCVAM

*Interagency Coordinating Committee on  
the Validation of Alternative Methods*



## Overview of the ICCVAM Evaluation of *In Vitro* Pyrogen Test Methods

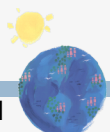
Richard McFarland, Ph.D., M.D.  
Chair, ICCVAM-Pyrogenicity Working Group

June 12, 2007  
SACATM Meeting  
Marriott Bethesda North Conference Center  
Bethesda, MD



# What Are Pyrogens and Where Do They Come From?

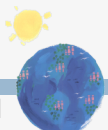
- Pyrogens increase body temperature by inducing leukocytes to release pro-inflammatory cytokines (e.g., interleukin [IL]-1, IL-6, and tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ]) that act as endogenous pyrogens
- Pyrogens originate from a variety of sources
  - Released from microbiological organisms (i.e., bacteria, viruses, and fungi) during cell death or following immunological attack
    - One of the most potent pyrogenic materials is bacterial endotoxin, a component of the outer cell wall of Gram-negative bacteria
  - Also may be found in processing and packaging materials, chemicals, raw materials, or equipment used during manufacturing of parenteral drugs or medical devices



# Why Pyrogen Testing?

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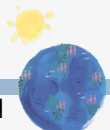
- **To protect public health:**
  - **Risk Management**
    - **To prevent endotoxin or non-endotoxin pyrogen-contaminated products (i.e., parenteral pharmaceuticals, biologicals, medical devices) from being introduced into humans or animals**



# Currently Accepted Pyrogen Tests

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- Rabbit Pyrogen Test (RPT)
  - **USP23 NF28<151>**
  - **Measures the temperature rise in rabbits injected with a test substance**
- Bacterial Endotoxin Test (BET)
  - **USP23 NF28<85>**
  - **Also referred to as the *Limulus* Amebocyte Lysate (LAL) Test**
    - **Derived from the horseshoe crab (*Limulus polyphemus*)**
  - **Employs a serine-protease catalytic cascade activated by endotoxin to produce a positive signal**



# ***In Vitro* Pyrogen Test Methods: Submission to ICCVAM/NICEATM**

- In June 2005, ECVAM submitted background review documents (BRDs) for five methods to NICEATM for consideration as replacements for the RPT.
  - **The Human Whole Blood (WB)/Interleukin (IL)-1 In Vitro Pyrogen Test**
  - **The Human WB/IL-1 In Vitro Pyrogen Test: Application of Cryopreserved Human WB**
  - **The Human WB/IL-6 In Vitro Pyrogen Test**
  - **The Human Peripheral Blood Mononuclear Cell (PBMC)/IL-6 In Vitro Pyrogen Test**
  - **An Alternative In Vitro Pyrogen Test Using the Monocytoid Cell Line Mono Mac 6 (MM6)/IL-6**
- **Following a NICEATM pre-screen evaluation, a request for additional information/clarification was sent from ICCVAM/NICEATM to ECVAM.**
- **In March 2006, revised BRDs were submitted by ECVAM which were then used as the formal test method submission.**



# ICCVAM PWG

## ■ Environmental Protection Agency (EPA)

- Karen Hamernik, Ph.D.
- Louis Scarano
- Ayaad Assad, D.V.M., Ph.D.

## ■ Food and Drug Administration (FDA)

- David Hussong, Ph.D.
- Richard McFarland, Ph.D., M.D. (Chair)
- Leonard Schechtman, Ph.D.\*
- Mustafa Akkoyunlu, M.D., Ph.D.
- Raju Kammula, D.V.M., Ph.D., D.A.B.T.
- Abigail Jacobs, Ph.D.
- Penelope Rice, Ph.D.
- Kimberly Benton, Ph.D.
- Pankaj Amin

\* Retired from FDA, 1/07

## ■ Food and Drug Administration (FDA) (Con't)

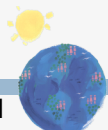
- Jiaqin Yao, Ph.D.
- Christopher Joneckis, Ph.D.
- Christine Anderson
- Daniela Verthelyi, M.D., Ph.D.
- Amy Rosenberg, M.D.
- Ramesh Panguluri

## ■ National Institute of Environmental Health Sciences (NIEHS)

- William Stokes, D.V.M., DACLAM
- Raymond Tice, Ph.D.

## ■ U.S. Department of Agriculture (USDA)

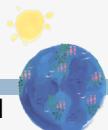
- Jodie Kulpa-Eddy, D.V.M.



# Pyrogen Test Method Review Activities

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- Jun 05:** Submissions for five *in vitro* pyrogen test methods received by NICEATM from the European Centre for the Validation of Alternative Methods (ECVAM)
- Sep 05:** ICCVAM-Pyrogenicity Working Group (PWG) established
- Dec 05:** PWG recommendations for peer review endorsed by SACATM
- 16 Dec 05:** *Federal Register* Notice published requesting data and nominations of experts for a peer review panel
- 13 Mar 06:** Revised ECVAM submission received in response to requests for information/clarification sent by NICEATM/ICCVAM
- 1 Dec 06:** ICCVAM draft Background Review Document (BRD) released to the peer review panel and public for review and comment
- 6 Feb 07:** Pyrogenicity Peer Review Panel Meeting convened
- 9 May 07:** Peer Review Panel Report published

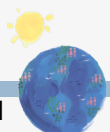


# ICCVAM Acceptance Criteria<sup>1</sup>

1. Fits into the regulatory testing structure
2. Adequately predicts the toxic endpoint of interest
3. Generates data useful for risk assessment
4. Adequate data available for specified uses
5. Robust and transferable
6. Time and cost-effective
7. Adequate animal welfare consideration (3Rs)

**The proposed use of the test method will provide for equivalent or improved protection of human and/or animal health, or the environment.**

<sup>1</sup>Adopted from: Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods; NIH Pub. No. 97-3981, 1997, NIEHS, Research Triangle Park, NC. <http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf>



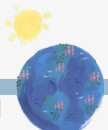
# ICCVAM Validation Criteria<sup>1</sup>

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1. Clear statement of proposed use
2. Biological basis/relationship to effect of interest
3. Formal detailed protocol
4. Reliability assessed
5. Relevance assessed
6. Limitations described
7. All data available for review
8. Data quality: *Ideally GLPs*
9. Independent scientific peer review

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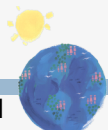
<sup>1</sup>Adopted from: Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods; NIH Pub. No. 97-3981, 1997, NIEHS, Research Triangle Park, NC. <http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf>



# ICCVAM Draft BRD

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- Provides a comprehensive review of available data and information regarding the usefulness and limitations of five alternative *in vitro* pyrogen test methods
  - Includes the submissions provided by ECVAM
- Describes the current validation status of the *in vitro* pyrogen test methods, including what is known about their relevance and reliability, the scope of the substances tested, and the availability of a standardized test method protocol for each test method
  - The test methods were reviewed for their ability to detect the presence of Gram-negative endotoxin when spiked into a variety of parenteral pharmaceuticals.



# Test Method Protocols Used in the Validation Study

- Although there are differences among the *in vitro* pyrogen test methods based predominantly on the cell type used, there are some basic steps that are consistent across all methods as follows:
  - The test substance is applied to the specific human-derived cells used in the *in vitro* test method (i.e., mixed with a suspension of cells).
  - The test substance is incubated with the cells for 16-24 hr.
  - The concentration of pro-inflammatory cytokines (e.g., IL-1 $\beta$ , IL-6) is quantified via a cytokine-specific enzyme-linked immunosorbent assay (ELISA) by comparison to a standard curve.
  - The endotoxin activity of a test substance is calculated by comparing the induced cytokine release with that induced by the endotoxin standard.
  - A product “passes” (i.e., is considered negative for endotoxin) if the endotoxin content is < 0.5 endotoxin units (EU)/mL.



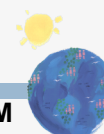
# Reference Data and Prediction Model Used for the Performance Evaluation

- Historical data from 171 rabbits tested with endotoxin were obtained from a single laboratory.
  - There were no direct comparisons using the same test substances in the proposed *in vitro* test methods and the RPT.
- The historical RPT data were used to establish a threshold pyrogen dose (i.e., the endotoxin dose at which fever was induced in 50% of the rabbits), which was determined to be 5 EU/kg.
- Based on the largest allowable volume for injection in rabbits (10 mL/kg), the limit of detection that the *in vitro* pyrogen tests must meet was defined as 0.5 EU/mL.
- Therefore, a substance was considered pyrogenic if the mean response  $\geq 0.5$  EU/mL.



# Substances Tested in the Validation Study

- A total of 13 substances, each spiked with multiple concentrations of Gram-negative endotoxin, were included in the performance analysis.
  - Accuracy: 10 substances, each spiked with four concentrations of Gram-negative endotoxin
    - Discordant results reflect a failure of the in vitro test method(s) to identify Gram-negative endotoxin spiked into a test substance at the threshold concentration (0.5 EU/mL)
  - Reproducibility: Three substances, each spiked with three concentrations of Gram-negative endotoxin



# Test Method Accuracy

Test Method	Accuracy <sup>1</sup>	Sensitivity	Specificity	False Negative Rate	False Positive Rate
Cryo WB/IL-1	92% (110/120)	97% (75/77)	81% (35/43)	3% (2/77)	19% (8/43)
MM6/IL-6	93% (138/148)	96% (85/89)	90% (53/59)	5% (4/89)	10% (6/59)
PBMC/IL-6	93% (140/150)	92% (83/90)	95% (57/60)	8% (7/90)	5% (3/60)
PBMC/IL-6 (Cryo) <sup>2</sup>	87% (130/150)	93% (84/90)	77% (46/60)	7% (6/90)	23% (14/60)
WB/IL-6	92% (136/148)	89% (79/89)	97% (57/59)	11% (10/89)	3% (2/59)
WB/IL-1	81% (119/147)	73% (64/88)	93% (55/59)	27% (24/88)	7% (4/59)
WB/IL-1 (96-well plate method) <sup>3</sup>	93% (129/139)	99% (83/84)	84% (46/55)	1% (1/84)	16% (9/55)

Abbreviations: Cryo = Cryopreserved; IL-1 = Interleukin-1; IL-6 = Interleukin -6; MM6 = Mono Mac 6; PBMC = Peripheral blood mononuclear cells; WB = Whole blood

<sup>1</sup>Percentage (Number of correct runs/total number of runs)

<sup>2</sup>A modification of the PBMC/IL-6 test method using cryopreserved PBMCs.

<sup>3</sup>A modification of the WB/IL-1 test method using 96-well plates instead of tubes for the test substance incubation



# Interlaboratory Reproducibility (1)

Lab Comparison <sup>1</sup>	Agreement Between Laboratories <sup>1</sup>				
	WB/IL-1	Cryo WB/IL-1 <sup>3</sup>	WB/IL-6	PBMC/IL-6	MM6/IL-6
1 vs 2	92% (77/84) <sup>2</sup>	92% (11/12)	72% (78/108)	81% (87/108)	97% (105/108)
1 vs 3	77% (83/108)	92% (11/12)	75% (81/108)	86% (93/108)	89% (96/108)
2 vs 3	68% (57/84) <sup>2</sup>	92% (11/12)	97% (105/108)	89% (96/108)	86% (93/108)
Mean	79%	92%	81%	85%	90%
Agreement Across 3 Labs <sup>4</sup>	58% (167/288) <sup>2</sup>	92% (11/12)	72% (234/324)	78% (252/324)	86% (279/324)

<sup>1</sup>Data from three substances spiked with endotoxin (WHO-LPS 94/580 [E. coli O113:H10:K-]) at 0, 0, 0.5 and 1.0 EU/mL tested three times in three different laboratories, with the exception of Cryo WB/IL-1 (only the preliminary run from each laboratory used for analysis)

<sup>2</sup>Some of the runs did not meet the assay acceptance criteria and therefore were excluded from the analysis.

<sup>3</sup>For the Cryo WB/IL-1 test method, each substance tested only once in each laboratory.

<sup>4</sup>All possible combinations of runs among the 3 laboratories were compared (with the exception of Cryo WB/IL-1, which was only tested once in each laboratory, resulting in only one possible combination per substance).



# Interlaboratory Reproducibility (2)

Lab Comparison <sup>1</sup>	Agreement Between Laboratories <sup>1</sup>						
	WB/IL-1	WB/IL-1 (plate) <sup>2</sup>	Cryo WB/IL-1	WB/IL-6	PBMC/IL-6	Ccryo PBMC/IL-6 <sup>3</sup>	MM6/IL-6
1 vs 2	73% (35/48)	88% (37/42)	84% (38/45)	85% (41/48)	84% (42/50)	96% (48/50)	90% (45/50)
1 vs 3	82% (40/49)	90% (35/39)	88% (21/24)	85% (41/48)	86% (43/50)	76% (38/50)	90% (43/48)
2 vs 3	70% (33/47)	92% (43/47)	100% (25/25)	88% (44/50)	90% (45/50)	80% (40/50)	83% (40/48)
Mean	75%	90%	91%	86%	87%	84%	88%
Agreement Across 3 Labs <sup>4</sup>	57% (27/47)	85% (33/39)	88% (21/24)	79% (38/48)	80% (40/50)	76% (38/50)	81% (39/48)

<sup>1</sup>Data from 10 substances spiked with endotoxin (WHO-LPS 94/580 [E. coli O113:H10:K-]) at 0, 0.25, 0.5, 0.5, and 1.0 EU/mL tested once in three different laboratories

<sup>2</sup>A modification to the WB/IL-1 test method protocol using 96-well plates instead of tubes for the incubation step.

<sup>3</sup>A modification to the PBMC/IL-6 test method protocol using cryopreserved PBMCs instead of fresh PBMCs.



# ICCVAM Draft Test Method Recommendations

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- Based on the information contained in the BRD, ICCVAM developed draft recommendations on:
  - Test method uses
  - Performance standards
  - Test method protocols
  - Future studies

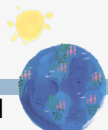


# ICCVAM Draft Recommendations (1)

## ■ **Proposed test method uses**

- *There is sufficient information, based on validation studies with a limited number of pharmaceuticals, to substantiate the use of these test methods (PBMC/IL-6, Cryo WB/IL-1 [96 well plate method], WB/IL-6, and MM6/IL-6) for the detection of pyrogenicity mediated by Gram-negative endotoxin in materials that are currently tested in the RPT, subject to product-specific validation to demonstrate equivalency<sup>1</sup>.*
- *While the scientific basis of these test methods suggests that they have the capability to detect pyrogenicity produced by a wider range of pyrogens (i.e., those mediated by non-endotoxin sources), there is insufficient data to support this broader application.*

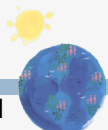
<sup>1</sup>*Equivalent methods can be regulated under 21CFR610.9 as alternatives to the currently accepted test method(s).*



# ICCVAM Draft Recommendations (2)

## ■ Performance Standards

- Essential test method components
  - Structural, functional, and procedural elements that should be included in the protocol of a mechanistically and functionally similar proposed test method.
- Reference Substances
  - Gram-negative endotoxin that has been spiked into each of the 10 substances that were tested in the ECVAM validation study.
- Accuracy and reliability values
  - When evaluated using the minimum list of recommended reference substances, the proposed test method should have performance characteristics that are comparable to the performance of the validated in vitro pyrogen test methods.
  - The reliability of the proposed test method for the reference substances should be comparable to or better than that of the validated in vitro pyrogen test methods.



# ICCVAM Draft Recommendations (3)

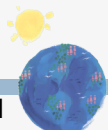
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## ■ Test method protocols

- Based on those protocols used in the ECVAM validation study

## ■ Future studies

- To further the use of these test methods, additional studies that include a broader range of pyrogenic materials are recommended.
  - Should use the recommended protocols
  - For a direct comparison between the in vitro pyrogen test(s) and the RPT, such studies should include parallel RPT testing.




# ICCVAM Peer Review Panel Meeting

**Independent Scientific Peer Review: Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products**

February 6, 2007 | Natcher Conference Center | NIH Campus  
8:30 a.m. - 5:00 p.m. | Conference Rooms E1/E2 | Bethesda, MD

**ICCVAM**  
Interagency Coordinating Committee  
on the Validation of Alternative Methods

**NICEATM**  
National Toxicology Program Interagency Center  
for the Evaluation of Alternative Toxicological Methods



IL-1 $\beta$  IL-6

**ICCVAM Agencies:**  
Agency for Toxic Substances and Disease Registry • Consumer Product Safety Commission • Department of Agriculture •  
Department of Defense • Department of Energy • Department of the Interior • Department of Transportation • Environmental  
Protection Agency • Food and Drug Administration • National Cancer Institute • National Institute of Environmental Health  
Sciences • National Institutes of Health, Office of the Director • National Institute for Occupational Safety and Health •  
National Library of Medicine • Occupational Safety and Health Administration

**NIHES**  
National Institute of  
Environmental Health Sciences

**NTP**  
National Toxicology Program  
U.S. Department of Health and Human Services

**ICCVAM**  
NICEATM

- **February 6, 2007**
  - NIH, Bethesda, MD
- **Expert Scientific Panel**
  - 13 scientists
  - 5 countries
- **Range of expertise includes:**
  - Immunology
  - Microbiology
  - In vivo and in vitro pyrogen testing
  - Biostatistics
  - Test method validation



# ICCVAM Charges to the Peer Panel

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- **Review the ICCVAM Draft In Vitro Pyrogen Test Methods Background Review Document (BRD) for completeness, and identify any errors or omissions in the BRD**
- **Evaluate the information in the draft BRD to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM Submission Guidelines 2003) have been appropriately addressed**
- **Consider the ICCVAM draft test method recommendations for the following and comment on the extent to which are supported by the information provided in the BRD**
  - **Proposed test method use**
  - **Proposed recommended standardized protocols**
  - **Proposed test method performance standards**
  - **Proposed future studies**

